



Mesenchymal Stem Cells Induce Suppressive Macrophages through Phagocytosis in a Mouse Model of Asthma

Faouzi Braza, Stéphanie Dirou, Virginie Forest, Vincent Sauzeau, Dorian Hassoun, Julie Chesné, Marie-aude Cheminant-muller, Christine Sagan, Antoine Magnan, Patricia Lemarchand

► To cite this version:

Faouzi Braza, Stéphanie Dirou, Virginie Forest, Vincent Sauzeau, Dorian Hassoun, et al.. Mesenchymal Stem Cells Induce Suppressive Macrophages through Phagocytosis in a Mouse Model of Asthma. STEM CELLS, 2016, 10.1002/stem.2344 . inserm-01277384

HAL Id: inserm-01277384

<https://www.hal.inserm.fr/inserm-01277384>

Submitted on 22 Feb 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Running head: Mesenchymal stem cell phagocytosis by macrophages

**Mesenchymal stem cells induce suppressive macrophages through phagocytosis
in a mouse model of asthma**

Faouzi Braza^{1,2,3*}, Stéphanie Dirou^{1,2,3,4*}, Virginie Forest^{1,2,3*}, Vincent Sauzeau^{1,2,3}, Dorian Hassoun^{1,2,3}, Julie Chesné^{1,2,3}, Marie-Aude Cheminant-Muller^{1,2,3}, Christine Sagan^{1,2,3,4}, Antoine Magnan^{1,2,3,4} and Patricia Lemarchand^{1,2,3,4}

¹ INSERM, UMR1087, l'institut du thorax, Nantes, F-44000 France;

² CNRS, UMR 6291, Nantes, F-44000 France;

³ Université de Nantes, Nantes, F-44000 France;

⁴ CHU de Nantes, Nantes, F-44000 France;

* Equal contributors

Author's contributions :

Faouzi Braza contributed to experiments, data acquiring and analysis, writing of the manuscript

Stéphanie Dirou contributed to experiments, writing of the manuscript

Virginie Forest contributed to the study design, experiments, data acquiring and analysis, writing of the manuscript

Vincent Sauzeau contributed to ex vivo airway smooth muscle contraction experiments

Dorian Hassoun contributed to lung function experiments

Julie Chesne contributed to experiments

Marie-Aude Cheminant-Muller contributed to animal experiments

Christine Sagan contributed to histological data analysis

Antoine Magnan contributed to data analysis and writing of the manuscript

Patricia Lemarchand contributed to the study design, data analysis and writing of the manuscript

Corresponding author: Patricia Lemarchand, MD, PhD,

INSERM, UMR1087/CNRS UMR 6294, l'institut du thorax, IRS-UN, 8 quai Moncousu, BP 70721, 44007 Nantes cedex 1, France,

Phone: +33(0)2-28-08-01-33

Fax: +33(0)2-28-08-01-30

Email: patricia.lemarchand@univ-nantes.fr

Keywords

house dust mite asthma, mesenchymal stem cells, M2 macrophage, airway hyper-responsiveness, phagocytosis, airway smooth muscle contraction.

Abstract

Mesenchymal stem cell (MSC) immunosuppressive functions make them attractive candidates for anti-inflammatory therapy in allergic asthma. However the mechanisms by which they ensure therapeutic effects remain to be elucidated. In an acute mouse model of house dust mite (Der f)-induced asthma, one i.v. MSC injection was sufficient to normalize and stabilize lung function in Der f-sensitized mice as compared to control mice. MSC injection decreased *in vivo* airway responsiveness and decreased *ex vivo* carbachol-induced bronchial contraction, maintaining bronchial expression of the inhibitory type 2 muscarinic receptor. To evaluate *in vivo* MSC survival, MSCs were labelled with PKH26 fluorescent marker prior to i.v. injection, and 1 to 10 days later total lungs were digested to obtain single-cell suspensions. 91.5 ± 2.3% and 86.6 ± 6.3% of the recovered PKH26⁺ lung cells expressed specific macrophage markers in control and Der f mice respectively, suggesting that macrophages had phagocyted *in vivo* the injected MSCs. Interestingly, only PKH26⁺ macrophages expressed M2 phenotype, while the innate PKH26⁻ macrophages expressed M1 phenotype. Finally, the remaining 0.5% PKH26⁺ MSCs expressed 10 to 100 fold more COX-2 than before injection, suggesting *in vivo* MSC phenotype modification. Together, the results of this study indicate that MSCs attenuate asthma by being phagocyted by lung macrophages, which in turn acquire a M2 suppressive phenotype.

Introduction

Growing bodies of evidences support the potent role of mesenchymal stem cells (MSCs) in the suppression of exacerbated inflammation [1, 2]. Based on these observations, MSCs are now used in pre-clinical animal models and human clinical trials as an alternative therapeutic strategy to suppress deleterious immune responses in the context of acute graft-versus-host disease [3], autoimmunity [4, 5] and interestingly in a variety of lung disorders [6, 7]. Altogether, more than 150 clinical trials have been registered on the NIH clinical trial registry, including the injection of MSCs [8].

Allergic asthma is a chronic inflammatory disease characterized by airway hyper-responsiveness and deregulated inflammation in response to allergens. This pathology is essentially controlled by CD4⁺ T helper 2 (T_H2) lymphocytes, which cause cellular infiltration in the lungs, overproduction of mucus and hyper-responsiveness. Some recent reports have highlighted the therapeutic efficacy of MSC injection, in both acute and chronic mouse models of asthma, by notably inducing the production of TGF- β [9], IL-10 [10], IFN γ [11, 12], increasing Treg cell number [13], and modulating the T_H1-T_H2 immunological balance [11, 14]. However despite these observations, very little is known about the exact mechanisms mediated by MSCs in lung, as the largest part of injected cells does not integrate the lungs [9, 15]. The therapeutic MSC effect has been attributed in part to paracrine effectors [16] including interaction with macrophages [15] via the PGE2 pathway [17, 18].

In this study, we took advantage of an acute model of house dust mite asthma to evaluate the mechanisms responsible for therapeutic efficacy of MSCs. Our data demonstrate that a single injection of MSCs improved lung function, inhibited contractile response of bronchi, decreased lung inflammation, and MSCs were *in vivo* phagocytosed by lung macrophages which in turn acquired a M2 suppressor phenotype.

Materials and Methods

Protocol - Allergic asthma was induced in 6- to 8-week-old BALB/c mice (Charles River Laboratories) using total Der f extracts (*Dermatophagoïdes farinae*) provided by Stallergenes as previously described [19]. The Regional Ethical Committee for Animal Experiments of the Pays de la Loire (CEEA.2010.50) approved all animal protocols. In brief, mice were sensitized at day 0, 7, 14 and 21 by percutaneous application of 500µg of Der f extracts in 20µL of dimethylsulfoxide (DMSO, Sigma-Aldrich) on the ears, and challenged intranasally with 250µg of Der f extracts in 40µL PBS at day 27 and 34 (Fig. 1). Control mice received only percutaneous application of 20µL DMSO and were challenged intranasally with PBS. During the first Der f challenge at day 27, mice also received 5.10⁵ MSCs in 200µL of PBS via tail vein injection, or 200µL PBS only. Lung function was analyzed by invasive method (Flexivent[®]) to evaluate airway resistance, in response to increased doses of methacholine (0, 5, 10, 15, 20mg/mL), according to the manufacturer instructions. Data were normalized to the results at 0 mg/mL dose.

Bone marrow MSC culture - Total bone marrow was obtained from several BALB/c mice by flushing femurs and tibias with culture medium (alpha MEM with nucleosides, 10% FCS, 2mM L-Glutamin, 100U/mL penicillin, 100µg/mL streptomycin, 2ng/mL human FGF2 (AbCys)). Cells were cultured until confluence, detached by 0.5% trypsin-EDTA and passed for amplification. From passage 8, MSCs were characterized by flow cytometry (BD LSRII, BD Biosciences, BD FACSDiva[™] software) for specific markers: Sca-1-PE (D7), CD11b-PE (M1/70, eBiosciences), CD44-PE (IM7), CD29-FITC (Ha2/5), CD45-PE (30-F11) (BD Biosciences), and for adipogenic and osteogenic differentiation capacity (see supplementary Fig. 1).

Ex vivo airway smooth muscle contraction - 2 mm-long rings of primary bronchi were cut and mounted on a multichannel isometric myograph (Danish Myo Technology). The bronchi were maintained in an organ bath containing Krebs-Henseleit buffer at 37°C continuously aerated (95% O₂ and 5% CO₂). A pre-tension of 0,5mN was applied and maintained all along the protocol. After an equilibration period, cumulative dose-response curves in response to KCl were obtained. In a second time, cumulative dose-response curves were obtained in response to carbachol, an analogue of methacholine (Sigma Aldrich).

Lung histology –Mice were euthanized for histology analysis at day 28 and 37. Lungs were inflated via the trachea with OCT diluted ½ in PBS, cryopreserved and sliced into continuous 5µm thick cryosections. Immunolabelling was performed to detect leucocytes with anti-CD45 antibody (Becton Dickinson) and cell nuclei with DAPI (Sigma Aldrich). After fluorescence microscopy analysis, the same sections were secondary stained with Hematoxyline-Eosin for light microscopy analysis.

Bronchoalveolar lavage and lung digestion - Bronchoalveolar lavage fluid (BAL) was obtained by instilling intra-tracheally 1mL PBS until lungs were inflated uniformly, followed by slow aspiration. BAL was centrifuged, and cells were counted and labelled for flow cytometry. After blood cardiac puncture, lungs were removed, minced into small pieces, and digested in 10mL PBS with 3mg/mL collagenase type II (Gibco) and 3mg/mL DNase I (Roche) at 37°C for 1 hour under agitation, to obtain lung single-cell suspension. Lung suspensions were filtered through a 40µm nylon filter. Red blood cells were lysed (BD FACS Lysing Solution 1X (Becton Dickinson)) and the remaining cells were washed, counted and labelled for flow cytometry or lysed for RNA extraction.

Flow cytometry – BAL cells were stained for multiparameter flow cytometric analysis. Ly6G-PercP.Cy5.5 (1A8), CD8-APC-H7 (53-6.7) (BD Biosciences), CD3-APC (145.2C11), CD19-PeCy7 (1D3), F4/80-FITC (BM8) (eBioscience), CCR3-PE (83101, R&D systems), and DAPI were used to identify BAL-infiltrating cells, as previously described [20]. Briefly, stained cells were acquired on a BD LSR™ II (BD Biosciences) and analyzed using BD FACSDiva™ software (BD Biosciences). Dead cells were excluded using DAPI. After gating on live cells, polymorphonuclear neutrophils (Ly6G^{hi} F4/80⁻ cells), macrophages (large, Ly6G⁻ F4/80⁺ cells), and eosinophils (F4/80⁻, Ly6G⁻, CCR3⁺ cells) were identified. Lymphocytes were identified as follows: forward scatter (FSC)^{lo}, side scatter (SSC)^{lo}, F4/80⁻, Ly6G⁻ and CCR3⁻ T (CD19⁻ CD3⁺) and B (CD19⁺ CD3⁻) cells were also identified.

To investigate the T helper responses, 8.10⁵ lung cells were transferred to a 96-well round-bottom plate and then stimulated for 5h with a mixture containing phorbol 12-myristate 13-acetate (PMA) (50ng/mL; Sigma-Aldrich) and ionomycin (500ng/mL; Sigma-Aldrich) together with either monensin (PMA + ionomycin + monensin; PIM) for T_H17 analysis (2mg/mL; BD Biosciences) or brefeldin A for T_H2 assessment (1mg/mL; BD Biosciences). For cytokine detection, the FcRs were blocked with mouse CD16/CD32 mAbs (eBioscience). Prior to surface-specific staining, the cells were stained with Fixable Viability Dye 450 (BD Biosciences) to exclude dead cells. The following antibodies were used for surface staining: CD3-PeCy7 (145-2C11) and CD8-APC-H7 (53-6.7) (BD Biosciences). The cells were then fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained intracellularly with anti-IL-4 (11B11; eBioscience), -IL-5 (TRFK5; BD Pharmingen), -IL-10 (JES5-16E3; BD Pharmingen), -IL-13 (eBio13A; eBioscience), -IL-17A (TC11-18H10; BD Biosciences) and -IFN γ (XMG1.2, BD Pharmingen) antibodies.

For *in vivo* MSC detection, MSCs cells were labelled just before injection with PKH26 according to manufacturer instructions (Red Fluorescent Cell Linker Kits for General Cell Membrane Labeling (Sigma Aldrich)). A total of 5.10⁵ PKH26⁺ MSCs were injected in mice.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Total lung cells were obtained 1 or 10 days later as indicated above and labelled with DAPI to select viable cells and CD11c-PeCy7 (N418) and F4/80-FITC (BM8) antibodies (eBioscience). Cells were sorted after gating for morphology (FSC-H vs FSC-A) and viability (negative for DAPI). On these gates, cells PKH26⁺ and PKH26⁻ were identified and further selected for cell sorting by F4/80 and CD11c expression. MSCs were evaluated and purified as PKH26⁺CD11c⁻F4/80⁻ cells (see supplementary Fig. 2) and counted in Malassez cell.

RNA isolation, Reverse Transcription, Real-Time PCR - Total RNA was extracted from lung homogenates, primary bronchi, and sorted cells using RNeasy mini and micro kit (Qiagen) according to manufacturer's instructions. Total RNA was reversely transcribed to cDNA using High Capacity cDNA Archive kit (Applied Biosystems). DNA templates were amplified by real-time quantitative PCR on the 7900 HT Fast Real-Time PCR System (ABI Prism, Applied Biosystems), using the Taqman® technology and commercial primers. Data were normalized to reference HPRT1 gene and were used for the $2^{-\Delta\Delta CT}$ calculation. $2^{-\Delta\Delta CT}$ corresponds to the ratio of each gene expression versus HPRT1 gene expression.

Statistical analysis – Comparisons of experimental values between two groups were analyzed using the Mann Whitney test. All statistical analysis were performed in GraphPad Prism v5. A p-value < 0.05 was considered statistically significant.

Results

Mesenchymal stem cells inhibit airway hyper-responsiveness and bronchoconstriction

To investigate the effect of MSC injection on allergic asthma, we used a well-established house dust mite (Der f)-induced asthma model [19] (Fig. 1). Mice were sensitized with percutaneous Der f extracts and then twice challenged with intranasal Der f extracts. A single i.v. injection of syngeneic MSCs was performed concomitantly to the first Der f challenge. One day after the second Der f challenge (day 35 post-sensitization) a decrease in airway hyper-responsiveness was observed in Der f mice injected with MSCs as compared to Der f mice injected with PBS (3.3 ± 1 in Der f + MSC mice vs 5.5 ± 2.1 cmHO ml⁻¹s⁻¹ in Der f + PBS mice at 20mg/mL methacholine, p=0.012, Fig. 2A), while MSC injection had no deleterious effect on lung function in control mice (3.3 ± 1.05 cmHO ml⁻¹s⁻¹ in control + MSC mice vs 3.03 ± 0.8 cmHO ml⁻¹s⁻¹ in control + PBS mice at 20mg/mL methacholine, p=0.3). This beneficial effect on lung function was stable 3 days after the second challenge (day 37 post-sensitization, 4.2 ± 1.9 in cmHO ml⁻¹s⁻¹ in Der f + MSC mice vs 8.2 ± 3.1 cmHO ml⁻¹s⁻¹ in Der f + PBS mice at 20mg/mL methacholine, p=0.03).

As exaggerated bronchial contraction in response to bronchoconstrictor agents is a specific feature of asthma, we evaluated if MSC i.v. injection altered the contractile bronchial response. Contractile force was quantified *ex vivo* on primary bronchi from Der f and control mice injected with MSCs or PBS, in response to KCl and carbachol (Fig. 2B). Consistent with our *in vivo* data on lung function, MSC injection during the first Der f challenge reduced carbachol-induced contraction when compared to Der f mice injected with PBS (2.1 ± 0.4 vs 0.97 ± 0.12 , p=0.002). Interestingly no difference was observed in KCl-induced contraction, suggesting that MSCs inhibited bronchoconstriction by regulating the muscarinic pathway. Der f mice injected with PBS displayed a decreased expression of the M2 muscarinic receptor in their bronchi, as compared to control mice injected with PBS or MSCs (Fig. 2C). In

contrast Der f mice injected with MSCs displayed M2 muscarinic receptor expression similar to that of control mice injected with PBS or MSCs. MSCs had no effect on M3 muscarinic receptor expression. Collectively these data suggest that MSCs mediated their protective role in asthma in part by modulating the airway contractile response.

Mesenchymal stem cells are located in lungs after i.v. injection

The presence of MSCs in lungs after i.v. injection was investigated using immuno-histology analysis. MSCs were labelled prior injection with PKH26, a fluorescent red marker which stably incorporates into cell membrane lipids. Successive immunofluorescence and histological analyses showed PKH26⁺ cell presence in alveolar and capillary walls, both in control and Der f mice (Fig. 3). PKH26⁺ cells were also located in inflammatory infiltrates in Der f mice (Fig 3, E and F). In both groups PKH26⁺ cells were still detected 10 days after MSC injection (Fig 3, C-D and G-H).

Quantification of mesenchymal stem cells in lungs after i.v injection

One day after injection of PKH26⁺ labelled MSCs in control and Der f mice, lungs were removed and digested, and lung cell suspensions were evaluated for the presence of PKH26⁺ cells using flow cytometry (Fig. 4A, upper panel). A total of $4 \pm 0.5 \%$ and $2.8 \pm 0.5 \%$ lung cells were identified as PKH26⁺ in control and Der f mice, respectively (n=6 in each group). Surprisingly, while $5 \cdot 10^5$ PKH26⁺ MSCs had been injected, the absolute number of PKH26⁺ cells detected in the lungs was $65 \pm 35 \cdot 10^3$ and $58 \pm 26 \cdot 10^3$ cells in control and Der f mice, respectively, suggesting that a high proportion of injected MSCs were still present in the lungs (13 ± 7 and $11,6 \pm 5\%$ in control and Der f mice, respectively). To evaluate if these cells were in fact macrophages, PKH26⁺ cells were co-labelled in lung cell suspensions with antibodies specific for macrophages (CD11c/F4/80). Importantly, two cell populations were easily distinguished into PKH26⁺ cell gate (Fig. 4A, lower panel), including a large majority of

PKH26⁺ cells positive for CD11c/F4/80 macrophage markers ($91.5 \pm 2.3\%$ and $86.6 \pm 6.3\%$ in control (n=6) and Der f (n=6) mice, respectively), and a minority of PKH26⁺ cells negative for CD11c/F4/80 macrophage markers ($4.27 \pm 0.7\%$ (n=6) and $5.74 \pm 1.47\%$ in control (n=6) and Der f mice (n=8), respectively). The absolute number of PKH26⁺CD11c⁺F4/80⁺ cells was 23 ± 15.10^3 cells and 27 ± 7.10^3 cells in control and Der f mice, accounting for $4.8 \pm 2.9\%$ and $5.5 \pm 1.4\%$ of 5.10^5 PKH26⁺ injected MSCs (Fig. 4C). The absolute number of PKH26⁺CD11c⁻F4/80⁻ cells was 763 ± 1046 and 2506 ± 2446 in control and Der f mice, represented $0.15 \pm 0.2\%$ and $0.5 \pm 0.5\%$ of 5.10^5 PKH26⁺ injected MSCs (Fig. 4D). Absolute cell numbers of both cell populations were not significantly different between both groups, suggesting that airway inflammation in sensitized mice did not increase cell engraftment.

Both populations of PKH26⁺ cells were then sorted using CD11c and F4/80 markers, and placed separately into cell culture plates in MSC culture medium. After 31 days of culture (Fig. 4B), PKH26⁺CD11c⁻F4/80⁻cells proliferated and expressed MSC phenotype (Sca-1⁺, CD29⁺, CD44⁺, CD45⁻, CD11b⁻; supplementary Fig. 3), confirming that these cells were injected PKH26⁺ MSCs. In marked contrast, PKH26⁺CD11c⁺F4/80⁺ cells did not proliferate in culture (Fig. 4B) and still expressed macrophage specific markers (CD11c⁺/F4/80⁺) confirming that these cells were macrophages (supplementary Fig. 3). Interestingly, PKH26⁺CD11c⁻F4/80⁻ cells were also observed 10 days after i.v. injection (0.06% and $0.1 \pm 0.09\%$ of 5.10^5 PKH26⁺ injected MSCs in control (n=3) and Der f (n=2) mice respectively), suggesting a mid-term survival of a very small number of MSCs in the lungs of mice (Fig. 4D). Together these data suggest that the vast majority of PKH26⁺ injected MSCs had been phagocytosed by lung macrophages during the 24hr following i.v. injection.

Injection of mesenchymal stem cells decrease airway inflammation

Airway inflammation is another important feature of allergic asthma. The first challenge with Der f extracts in Der f mice induced the infiltration of both eosinophils and neutrophils in

BAL, associated with the expansion of lung lymphocytes (Fig. 5A). MSCs did not alter immunologic profile in control mice, suggesting no deleterious effect and no inflammation response due to MSC injection itself (Fig. 5A). Interestingly, 24 hours after the first Der f challenge (day 28), MSC injection inhibited airway inflammation with decreased absolute numbers of BAL neutrophils, eosinophils and lymphocytes. In parallel a decrease was observed in cytokine (IL-4, IL-5, IL-13 and IL-17) expression by lung CD4⁺ T cells (Fig. 5B) while no significant effect was observed to IFN γ and IL-10 expression. These data demonstrate that MSCs early constrained airway inflammation, however this suppressive effect declined after a second challenge (data not shown) despite the persistence of decreased hyper-responsiveness (Fig. 2A).

MSC injection induces lung macrophage polarization into suppressive phenotype

To evaluate whether phagocytosis of MSCs by lung macrophages could alter their function and explain MSC suppressive effects, total lung cells were obtained from control and Der f mice injected with PKH26-labelled MSCs as described above. Lung macrophages were then cell-sorted according to their specific co-expression of CD11c and F4/80, and divided into 2 sub-populations according to their differential PKH26 labelling. Both macrophage subpopulations were then evaluated for their gene expression in pro and anti-inflammatory cytokines. Interestingly, the functional profile of PKH26⁺ and PKH26⁻ macrophages was different (Fig. 6A). PKH26⁺ macrophages expressed higher mRNA levels of TGF β 1 and IL-10, two immunosuppressive cytokines that are typically increased in M2 macrophages [21]. In contrast, PKH26⁻ macrophages expressed M1 macrophage phenotype with higher levels of IL-6. No difference in TNF α mRNA expression was observed. Importantly, no difference in cytokine expression in lung macrophages was observed between control and Der f mice both injected with MSCs, suggesting that the induction of M2 macrophage phenotype was due to MSC injection and not to Der f sensitization. We then evaluated if MSCs expressed *in vivo*

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

COX-2, a cyclo-oxygenase which catalyzes the rate-limiting step of prostaglandin formation, including PGE-2, a central molecule necessary for M2 macrophage activation [22]. To answer this question, COX-2 gene expression was quantified in PKH26⁺CD11c⁻F4/80⁻ cells, as these cells were demonstrated above as being MSCs (Fig. 4B). Sorted PKH26⁺CD11c⁻F4/80⁻ cells expressed significantly higher mRNA levels of COX-2 than MSCs before their injection (Fig. 6B). The over-expression of COX-2 in MSCs was still observed 10 days after the first Der f challenge and MSC injection. No difference in COX-2 expression in PKH26⁺CD11c⁻F4/80⁻ cells was observed between control and Der f mice injected with MSCs, suggesting that the increase in COX-2 expression was due to MSC injection and not to Der f sensitization.

Discussion

Numerous studies on MSCs and asthma therapy showed that MSC i.v. injection attenuate airway inflammation and hyper-responsiveness [9, 11-13, 23, 24], but the lack of mechanistic explanation hampered this result, considering the very low quantity of cells that survive and graft in lung tissue after injection. In this study we showed that injected MSCs are *in vivo* phagocytosed by lung macrophages that, in turn, reprogram into an immunosuppressive phenotype.

Cell tracking after *in vivo* administration is one of the trickiest issues in cell therapy studies and results from lung studies remain controversial [25]. The use of GFP as a marker of stem cells obtained from bone marrow of GFP⁺ transgenic mice, can lead to errors in the presence of autofluorescent structures [26]. Microscopy-based methods even when coupled with immunohistochemistry-based cell visualization and characterization suffer from low sensitivity and problems associated with reliable interpretation of images [27]. To circumvent this issue, we injected MSCs labelled with PKH26 red fluorescent marker, and sorted PKH26⁺ cells by flow cytometry from digested total lungs, 1 to 10 days after MSC injection. Our initial results suggesting high lung MSC engraftment were similar to that observed using the same methodology in a model of rat lung injury [28]. Nevertheless, such high lung engraftment was in discrepancy with previous studies in numerous lung injury animal models of stem cell therapy [29, 30]. By using sophisticated microscopy methods such as fluorescence confocal deconvolution microscopy, the participation to the lungs of adult stem cells from bone marrow was evaluated between 0.1 and 1 % [31-34]. On the other hand, a critical role of macrophages has been suggested as immune effector in MSC therapy models. Macrophages play an important role in acute allergic lung inflammation [35, 36], and interaction with macrophages is necessary for an optimal therapeutic effect of MSCs [15]. Depletion of alveolar macrophages reverses the benefit of stem cell treatment [15] and

increases inflammation [36]. We thus raised the hypothesis that injected MSCs had been phagocytosed by macrophages. Using specific markers for macrophages we demonstrated that the sorted lung PKH26⁺ cells were indeed mostly macrophages, whereas a small number of characterized MSCs were observed (0.15 to 0.5% of MSC injected cells). This proportion of grafted MSCs in the lungs is in adequacy with the current literature, and showed no increase in cell engraftment despite lung inflammation, a result that has already been observed in other animal models [37]. On the other hand, the mechanisms by which such small MSC number suppresses airway responsiveness remain largely unexplained [38].

Recent observations suggest that under many conditions MSCs only transiently appear in injured tissues, but during their brief appearance they cross-talk with injured cells to limit tissue destruction or enhance repair by a variety of mechanisms [10, 38, 39]. Activated MSCs secrete COX-2, and as a result, MSCs increase PGE2 secretion that drives resident macrophages with an M1 proinflammatory phenotype toward an M2 anti-inflammatory phenotype [22]. M2 macrophages show high phagocytic activity, they produce low amounts of pro-inflammatory cytokines and higher amounts of the anti-inflammatory cytokine IL-10, and they contribute to inflammation resolution [21]. In our study, MSC injection induced lung macrophage polarization into M2 suppressive phenotype, without inducing an increase in macrophage number, as already observed in ovalbumin-induced allergic model [40]. In turn, injected MSCs expressed 10 to 100 fold more COX-2 than before injection, suggesting that MSC phenotype alteration occurred after *in vivo* injection, in presence or absence of inflammation. Interestingly, MSCs need to be viable when injected, as delivery of PFA-fixed MSCs to allergen-sensitized mice resulted in severe pathology [13]. Importantly, the induction of M2 suppressive phenotype was observed in our study only in macrophages that had phagocytosed PKH26⁺ MSCs and not in “naive” macrophages (*i.e.*, PKH26⁻ macrophages), suggesting that M2 suppressive phenotype was induced by MSC phagocytosis and not by simple cross-talk between macrophages and MSCs. In summary these data demonstrate that

1 injected MSCs *in vivo* are phagocytosed in the lungs after i.v. injection, inducing a suppressive
2 macrophage cellular phenotype. By doing this, MSCs promote the establishment of a
3 tolerogenic microenvironment that might probably counterbalance T-cell mediated airway
4 inflammation.
5
6
7
8
9

10 It is strongly believed that MSCs achieve a therapeutic effect *in vivo* via paracrine action, in
11 particular the shedding of extracellular vesicles including exosomes and microvesicles [41].
12 Under standard *in vitro* culture conditions, Phinney et al recently demonstrated that MSCs
13 undergo mitophagy and use microvesicles to unload mitochondria, which are engulfed by
14 macrophages and re-utilized to increase bioenergetics [42]. In our study, to label MSCs prior
15 to *in vivo* injection we used PKH26 labelling, a fluorescent red marker which stably
16 incorporates into cell membrane lipids. Therefore, PKH26+ macrophages in lung suspensions
17 may be macrophages that have phagocytosed exosomes with PKH26+ membranes, without
18 phagocytosing whole MSCs. However, this mechanism cannot explain the low number of
19 surviving MSCs 24hr after *in vivo* administration (less than 0.5%), suggesting that MSC
20 phagocytosis by macrophages is, at least in part, responsible for low MSC surviving.
21
22
23
24
25
26
27
28
29
30
31
32
33
34

35 We chose a house dust mite mice allergic asthma model, because house dust mite is the main
36 indoor allergens responsible for human asthma, and this model being characterized by
37 abnormal T_H2 and T_H17 immune response as observed in patients with severe asthma [19].
38 MSC injection at day 0 before Der f sensitization did not prevent the alteration in respiratory
39 function during subsequent Der f challenges in our model (data not shown). This suggests that
40 MSC injection did not protect from sensitization to new allergen but could attenuate
41 deleterious reaction to repeated allergen exposure and prevent further asthma exacerbation
42 and remodeling. Similar to our results, MSC injection could modulate airway inflammation in
43 chronic asthma model when MSCs were administered during the installation of inflammation
44 [23].
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

MSC administration decreased significantly airway hyper-responsiveness induced by methacholine only in asthmatic Der f mice, and this result correlated with the reduction of *ex vivo* primary bronchial contraction. Furthermore MSC injection increased M2 muscarinic receptor expression in asthmatic mice. Low or dysfunctional M2 muscarinic receptor activity has been associated with airway hyper-responsiveness in mice [43]. M2 muscarinic receptor inhibited the release of acetylcholine, a cholinergic neurotransmitter typically increased in asthmatic patients, promoting airway contraction [44]. MSC injection induced a diminution of eosinophils and IL-5, two factors influencing the M2 muscarinic receptor expression [45, 46]. In summary, our data suggest that the mechanism of airway hyper-responsiveness decrease by MSC injection was not limited to a modification of inflammation response as observed in numerous studies [47], but was also linked to a modification of airway contractility.

Conclusion:

Cell engraftment after i.v injection to repair injured tissue has always been a challenge since the beginning of cell therapy studies. We showed in a mouse model of asthma that most of injected MSCs, not only do not engraft into lungs but are *in vivo* phagocytosed by lung macrophages which, in turn, acquire a M2 suppressive phenotype. This mechanism was sufficient to decrease airway responsiveness and to observe MSC anti-inflammatory efficacy. As MSCs are being used for their immunomodulatory properties in the treatment of a number of immune-based disorders [48], further studies on such mechanisms in other models **and to unravel early *in vivo* interactions between MSCs and lung macrophages** are warranted.

Acknowledgments : This work was supported by a grant from Institut de Recherche en Santé Respiratoire des Pays de la Loire (France). The authors acknowledge the Experimental Therapy Unit facility, the Cellular and Tissular Imaging Core Facility of Nantes University (MicroPICell), and the Flow Cytometry and Cell Sorting facility (Cytocell).

Disclosure of Potential Conflicts of interest : The authors have declared that no conflict of interest exists.

References

1. Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood. 2007;110(10):3499-506.

2. Le Blanc K, Ringden O. Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med. 2007;262(5):509-25.

3. Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 2004;363(9419):1439-41.

4. Fiorina P, Jurewicz M, Augello A, et al. Immunomodulatory function of bone marrow-derived mesenchymal stem cells in experimental autoimmune type 1 diabetes. J Immunol. 2009;183(2):993-1004.

5. Rafei M, Campeau PM, Aguilar-Mahecha A, et al. Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner. J Immunol. 2009;182(10):5994-6002.

6. Inamdar AC, Inamdar AA. Mesenchymal stem cell therapy in lung disorders: pathogenesis of lung diseases and mechanism of action of mesenchymal stem cell. Exp Lung Res. 2013;39(8):315-27.

7. Weiss DJ. Concise review: current status of stem cells and regenerative medicine in lung biology and diseases. Stem Cells. 2014;32(1):16-25.

8. <https://clinicaltrials.gov/ct2/results?intr=injection+of+mesenchymal+stem+cells> CgWS.

9. Nemeth K, Keane-Myers A, Brown JM, et al. Bone marrow stromal cells use TGF-beta to suppress allergic responses in a mouse model of ragweed-induced asthma. Proc Natl Acad Sci U S A. 2010;107(12):5652-7.

10. Ionescu LI, Alphonse RS, Arizmendi N, et al. Airway delivery of soluble factors from plastic-adherent bone marrow cells prevents murine asthma. *Am J Respir Cell Mol Biol*. 2012;46(2):207-16.
11. Goodwin M, Sueblinvong V, Eisenhauer P, et al. Bone marrow-derived mesenchymal stromal cells inhibit Th2-mediated allergic airways inflammation in mice. *Stem Cells*. 2011;29(7):1137-48.
12. Park HK, Cho KS, Park HY, et al. Adipose-derived stromal cells inhibit allergic airway inflammation in mice. *Stem Cells Dev*. 2010;19(11):1811-8.
13. Kavanagh H, Mahon BP. Allogeneic mesenchymal stem cells prevent allergic airway inflammation by inducing murine regulatory T cells. *Allergy*. 2011;66(4):523-31.
14. Ou-Yang HF, Huang Y, Hu XB, et al. Suppression of allergic airway inflammation in a mouse model of asthma by exogenous mesenchymal stem cells. *Exp Biol Med (Maywood)*. 2011;236(12):1461-7.
15. Mathias LJ, Khong SM, Spyroglou L, et al. Alveolar macrophages are critical for the inhibition of allergic asthma by mesenchymal stromal cells. *J Immunol*. 2013;191(12):5914-24.
16. Lee JW, Fang X, Krasnodembskaya A, et al. Concise review: Mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. *Stem Cells*. 2011;29(6):913-9.
17. Nemeth K, Leelahavanichkul A, Yuen PS, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med*. 2009;15(1):42-9.
18. Ylostalo JH, Bartosh TJ, Coble K, et al. Human mesenchymal stem/stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype. *Stem Cells*. 2012;30(10):2283-96.

19. Chesne J, Braza F, Chadeuf G, et al. Prime role of IL-17A in neutrophilia and airway smooth muscle contraction in a house dust mite-induced allergic asthma model. *J Allergy Clin Immunol.* 2015;135(6):1643-5 e5.

20. Amu S, Saunders SP, Kronenberg M, et al. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *J Allergy Clin Immunol.* 2010;125(5):1114-24 e8.

21. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest.* 2012;122(3):787-95.

22. Prockop DJ. Concise review: two negative feedback loops place mesenchymal stem/stromal cells at the center of early regulators of inflammation. *Stem Cells.* 2013;31(10):2042-6.

23. Bonfield TL, Koloze M, Lennon DP, et al. Human mesenchymal stem cells suppress chronic airway inflammation in the murine ovalbumin asthma model. *Am J Physiol Lung Cell Mol Physiol.* 2010;299(6):L760-70.

24. Sun YQ, Deng MX, He J, et al. Human pluripotent stem cell-derived mesenchymal stem cells prevent allergic airway inflammation in mice. *Stem Cells.* 2012;30(12):2692-9.

25. Loebinger MR, Aguilar S, Janes SM. Therapeutic potential of stem cells in lung disease: progress and pitfalls. *Clin Sci (Lond).* 2008;114(2):99-108.

26. Swenson ES, Price JG, Brazelton T, et al. Limitations of green fluorescent protein as a cell lineage marker. *Stem Cells.* 2007;25(10):2593-600.

27. Griesenbach U, Alton EW. Progress in gene and cell therapy for cystic fibrosis lung disease. *Curr Pharm Des.* 2012;18(5):642-62.

28. Curley GF, Ansari B, Hayes M, et al. Effects of intratracheal mesenchymal stromal cell therapy during recovery and resolution after ventilator-induced lung injury. *Anesthesiology.* 2013;118(4):924-32.

29. Sinclair K, Yerkovich ST, Chambers DC. Mesenchymal stem cells and the lung. *Respirology*. 2013;18(3):397-411.
30. Zhu YG, Hao Q, Monsel A, et al. Adult stem cells for acute lung injury: remaining questions and concerns. *Respirology*. 2013;18(5):744-56.
31. Kotton DN, Fabian AJ, Mulligan RC. Failure of bone marrow to reconstitute lung epithelium. *Am J Respir Cell Mol Biol*. 2005;33(4):328-34.
32. Krause DS. Engraftment of bone marrow-derived epithelial cells. *Ann N Y Acad Sci*. 2005;1044:117-24.
33. Bruscia EM, Ziegler EC, Price JE, et al. Engraftment of donor-derived epithelial cells in multiple organs following bone marrow transplantation into newborn mice. *Stem Cells*. 2006;24(10):2299-308.
34. Loi R, Beckett T, Goncz KK, et al. Limited restoration of cystic fibrosis lung epithelium in vivo with adult bone marrow-derived cells. *Am J Respir Crit Care Med*. 2006;173(2):171-9.
35. Mathie SA, Dixon KL, Walker SA, et al. Alveolar macrophages are sentinels of murine pulmonary homeostasis following inhaled antigen challenge. *Allergy*. 2015;70(1):80-9.
36. Zaslona Z, Przybranowski S, Wilke C, et al. Resident Alveolar Macrophages Suppress, whereas Recruited Monocytes Promote, Allergic Lung Inflammation in Murine Models of Asthma. *J Immunol*. 2014;193(8):4245-53.
37. Forest VF, Tirouvanziam AM, Perigaud C, et al. Cell distribution after intracoronary bone marrow stem cell delivery in damaged and undamaged myocardium: implications for clinical trials. *Stem Cell Res Ther*. 2010;1(1):4.
38. Prockop DJ, Oh JY. Medical therapies with adult stem/progenitor cells (MSCs): a backward journey from dramatic results in vivo to the cellular and molecular explanations. *J Cell Biochem*. 2012;113(5):1460-9.

39. Prockop DJ. Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. *Mol Ther.* 2009;17(6):939-46.

40. Song X, Xie S, Lu K, et al. Mesenchymal stem cells alleviate experimental asthma by inducing polarization of alveolar macrophages. *Inflammation.* 2015;38(2):485-92.

41. Lee C, Mitsialis SA, Aslam M, et al. Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation.* 2012;126(22):2601-11.

42. Phinney DG, Di Giuseppe M, Njah J, et al. Mesenchymal stem cells use extracellular vesicles to outsource mitophagy and shuttle microRNAs. *Nat Commun.* 2015;6:8472.

43. Castro JM, Resende RR, Mirotti L, et al. Role of m2 muscarinic receptor in the airway response to methacholine of mice selected for minimal or maximal acute inflammatory response. *Biomed Res Int.* 2013;2013:805627.

44. Hirshman CA, Lande B, Croxton TL. Role of M2 muscarinic receptors in airway smooth muscle contraction. *Life Sci.* 1999;64(6-7):443-8.

45. Elbon CL, Jacoby DB, Fryer AD. Pretreatment with an antibody to interleukin-5 prevents loss of pulmonary M2 muscarinic receptor function in antigen-challenged guinea pigs. *Am J Respir Cell Mol Biol.* 1995;12(3):320-8.

46. Jacoby DB, Gleich GJ, Fryer AD. Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M2 receptor. *J Clin Invest.* 1993;91(4):1314-8.

47. Srouf N, Thebaud B. Stem cells in animal asthma models: a systematic review. *Cytotherapy.* 2014;16(12):1629-42.

48. Wang Y, Chen X, Cao W, et al. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol.* 2014;15(11):1009-16.

Figure legends

Figure 1 : Allergic asthma protocol.

Mice were sensitized by percutaneous administrations of total house dust mite (Der f) extracts or DMSO for controls, once a week during 4 weeks, and challenged by intranasal instillations of Der f extracts or PBS for controls, once a week during 2 weeks. 5.10^5 MSCs in 200 μ L PBS or 200 μ L PBS in control mice were injected in tail vein during the first challenge at day 27. Analyses were performed at day 28 (24hr after MSC injection), at day 35 (1 day after the last challenge) or at day 37 (3 days after the last challenge).

Figure 2: Mesenchymal stem cells inhibit airway hyper-responsiveness and bronchoconstriction.

(A) Airway resistance was measured using Flexivent[®] after instillation of 0 to 20mg/mL methacholine, in control and asthmatic Der f mice that had received PBS (as a control) or 5.10^5 MSCs, respectively, at day 27 following first Der f sensitization. Measurements were realized at day 35 in control mice, and at day 35 and 37 following first Der f sensitization (1 or 3 days after the second challenge, respectively) in Der f mice. Data are means \pm SEM. (B) Contractile response of primary bronchi (2 mm-long rings) from control and Der f asthmatic mice injected with PBS or MSCs, stimulated with KCl or carbachol. Measurements were performed in 2 primary bronchi per each mouse at day 37. Data are means \pm SEM. (C) Relative mRNA expression levels of M2 and M3 muscarinic receptors, evaluated by real-time PCR in primary bronchi from control and Der f asthmatic mice injected with PBS or MSCs. mRNA transcription level was normalized to HPRT1 expression. Data are expressed as means \pm SD. ** $p < 0.01$ compared to control + PBS mice. Statistical differences were determined by a Mann Whitney test, ** $p < 0.01$; * $p < 0.05$.

Figure 3: Mesenchymal stem cells were located in alveolar walls and capillary walls after i.v. injection.

Each panel shows representative images of histological fluorescence analysis (A,C,E,G), followed by H&E staining (B,D,F,H) of the same lung sections, from control and asthmatic Der f mice sacrificed 24hr (Day 28) and 10 days (Day 37) after PKH26⁺ MSC injection. Smaller panels are zoomed images of the dotted rectangles. DAPI (blue) stained nuclei and anti-CD45 antibody (green) stained leucocytes. PKH26⁺ cells (arrow and red) were located in alveolar walls and capillary walls in both control and asthmatic mice, and in inflammatory infiltrates in asthmatic mice. *: alveolus. **: airway bronchiole. Scale bar: 50 μ m.

Figure 4: Characterization of the PKH26⁺ injected MSCs.

Lungs were removed and digested with collagenase in control or Der f mice, 24hr (Day 28) or 10 days (Day 37) after i.v. injection of PKH26⁺ labelled MSCs, to obtain lung single-cell suspensions. Lung cells were then labelled with DAPI, CD11c and F4/80 antibodies. (A) Representative example of flow cytometric analysis and sorting at day 28 in lung cell suspension from a control mouse injected with PKH26⁺ labelled MSCs. PKH26⁺ cells were identified in lung cell suspension (PKH26 vs FSC-H, upper panel). After gating on this PKH26⁺ cells, PKH26⁺ cells were sorted according to the presence or absence of expression of both CD11c and F4/80 macrophage markers (CD11c vs F4/80, low panel). (B) Representative images of cell plates containing PKH26⁺CD11c⁺F4/80⁺ and PKH26⁺CD11c⁻F4/80⁻ sorted cells and cultured separately in MSC culture medium. PKH26⁺CD11c⁺F4/80⁺sorted cells (upper panels) did not proliferate and had round shape morphology, whereas PKH26⁺CD11c⁻F4/80⁻ sorted cells (lower panels) proliferated, requiring 2 to 4 cell passages, and presented with long shape morphology. (C-D) Absolute numbers of PKH26⁺CD11c⁺F4/80⁺ sorted cells (C) and PKH26⁺CD11c⁻F4/80⁻ sorted cells (D), 24hr and

10 days after PKH26⁺ MSC injection. Statistical differences were determined by a Mann Whitney test, **p<0.01; * p<0.05, ns : not significant.

Figure 5: Injection of mesenchymal stem cells decreased airway inflammation

(A) Total number of macrophages (Mac), neutrophils (PMN), eosinophils (PNE) and lymphocytes (Lym), were obtained by flow cytometry on bronchoalveolar lavages at day 28 in control and Der f mice injected with PBS or with MSCs. Data are expressed as means \pm SD.

(B) Percentage of IL-4, IL-5, IL-13, IL-17, IFN γ , and IL-10 positive CD4⁺ T cells isolated from the lungs at day 28, from control and Der f mice injected with PBS or with MSCs. Data are expressed as means \pm SD. Statistical differences were determined by a Mann Whitney test, ** p<0.01; * p<0.05, ns : not significant.

Figure 6: MSC injection induced macrophage polarization into suppressive phenotype in the lungs

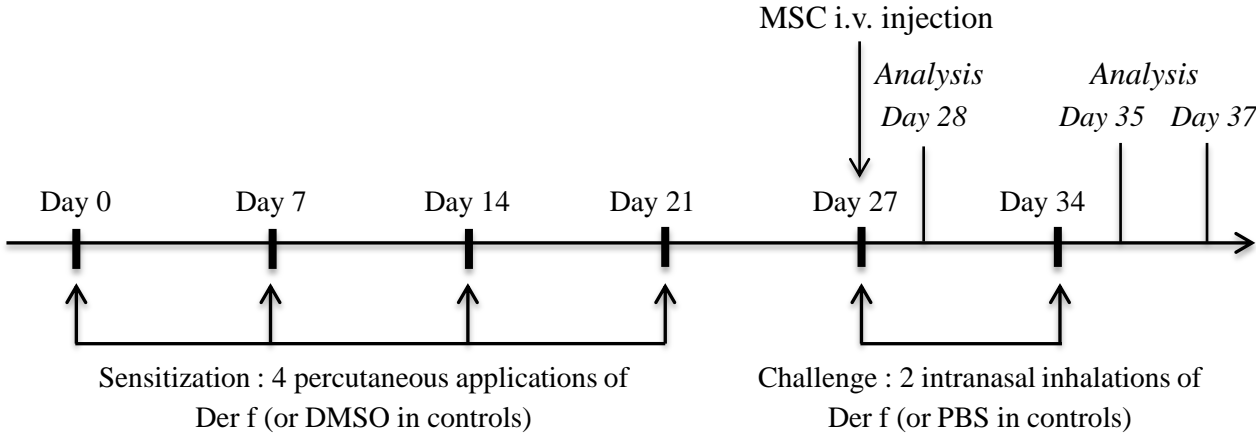
A) Relative mRNA expression levels of TGF β 1, IL-10, IL-6 and TNF α in PKH26⁺ (PKH26⁺CD11c⁺F4/80⁺) and PKH26⁻ (PKH26⁻CD11c⁺F4/80⁺) macrophages sorted from lung cell suspensions using flow cytometry. Measurements were performed at day 28 (24hr after PKH26⁺ MSC injection) in control (n=6 to 8) and Der f mice (n=5 to 6). (B) Relative COX-2 mRNA expression levels in MSCs *in vitro* just before injection (n=5), and in PKH26⁺CD11c⁻F4/80⁻ cells (PKH26⁺ MSCs) cells sorted from lung cell suspensions using flow cytometry. Measurements were performed in control mice (n=2 to 7) and in Der f mice (n=3 to 6) at day 28 (24hr after PKH26⁺ MSC injection) and day 37 (10 days after PKH26⁺ MSC injection). mRNA expression was expressed in logarithmic scale. mRNA transcription level was normalized with HPRT1 expression. Statistical differences were determined by a Mann Whitney test, ** p<0.01 and * p<0.05.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

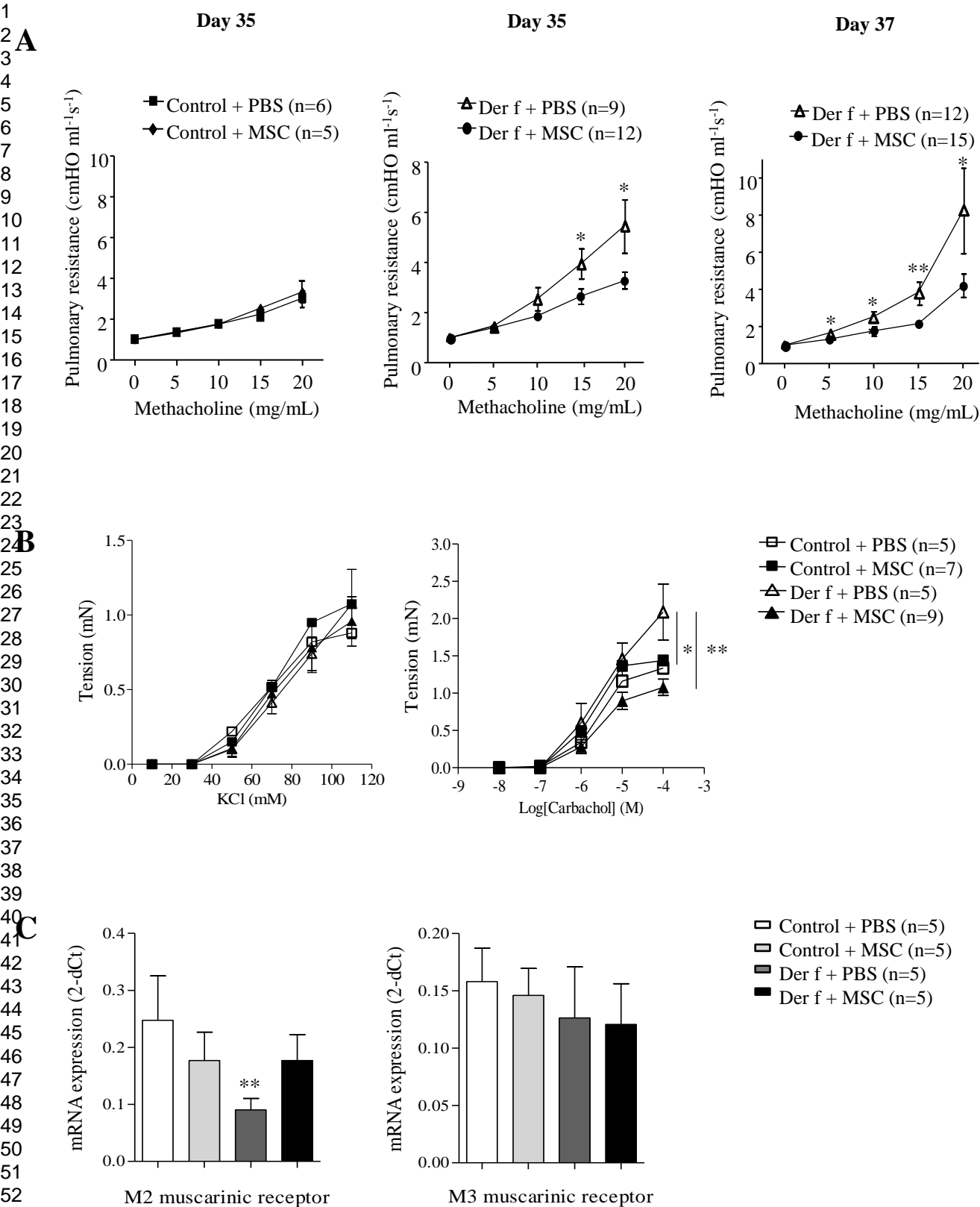
Graphical Abstract

Lung macrophages have been cell-sorted from lung cell suspensions, 24hr after intravenous injection of PKH26+ mesenchymal stem cells (MSCs). PKH26 staining is characterized by incorporation of red fluorescent molecules into the cell membrane and induces intense and reproducible fluorescence. Lung macrophages show PKH26+ (red) labelling, suggesting that they had *in vivo* phagocytosed the PKH26+ MSCs.

Figure 1



Stem Cells
Figure 2



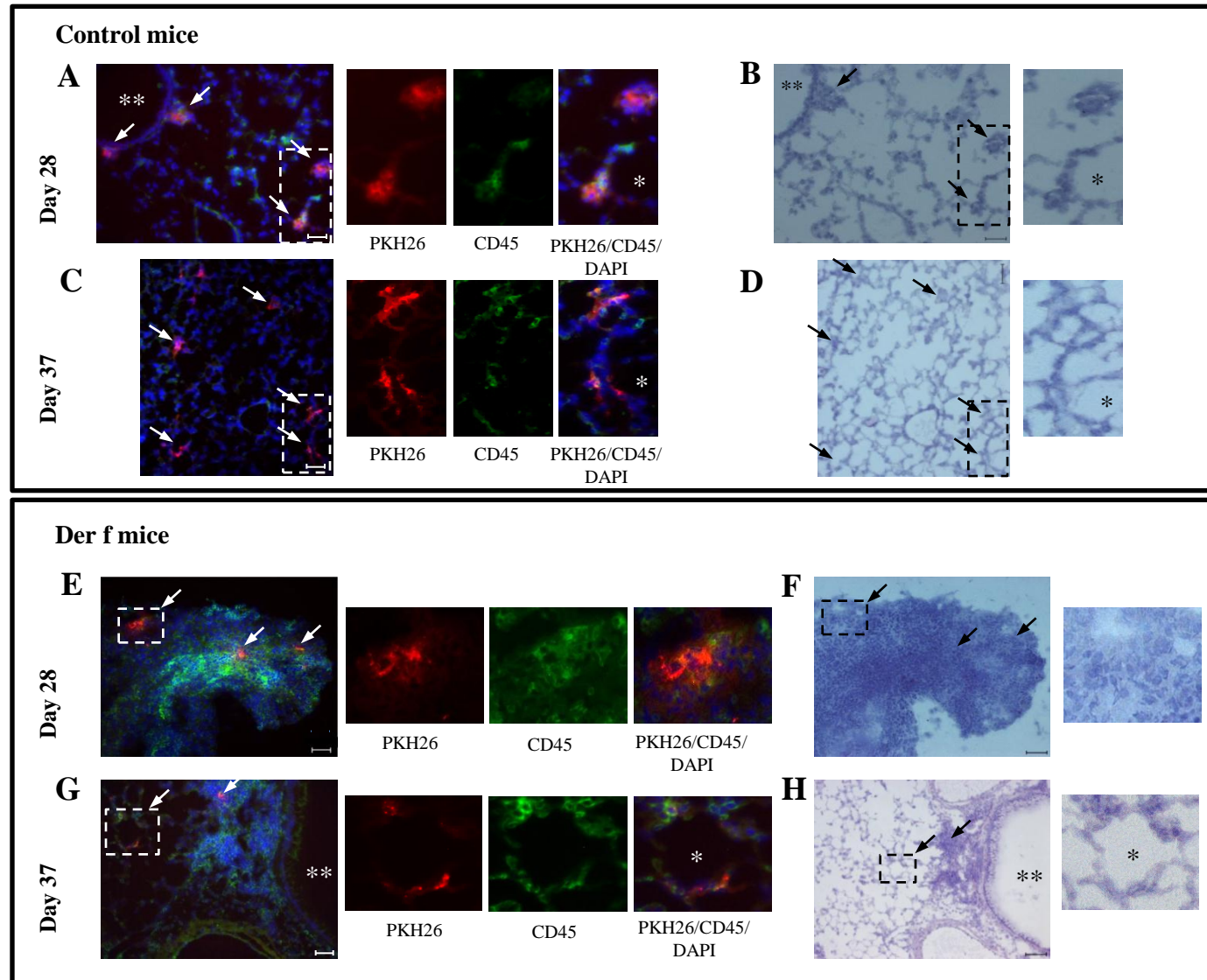


Figure 4

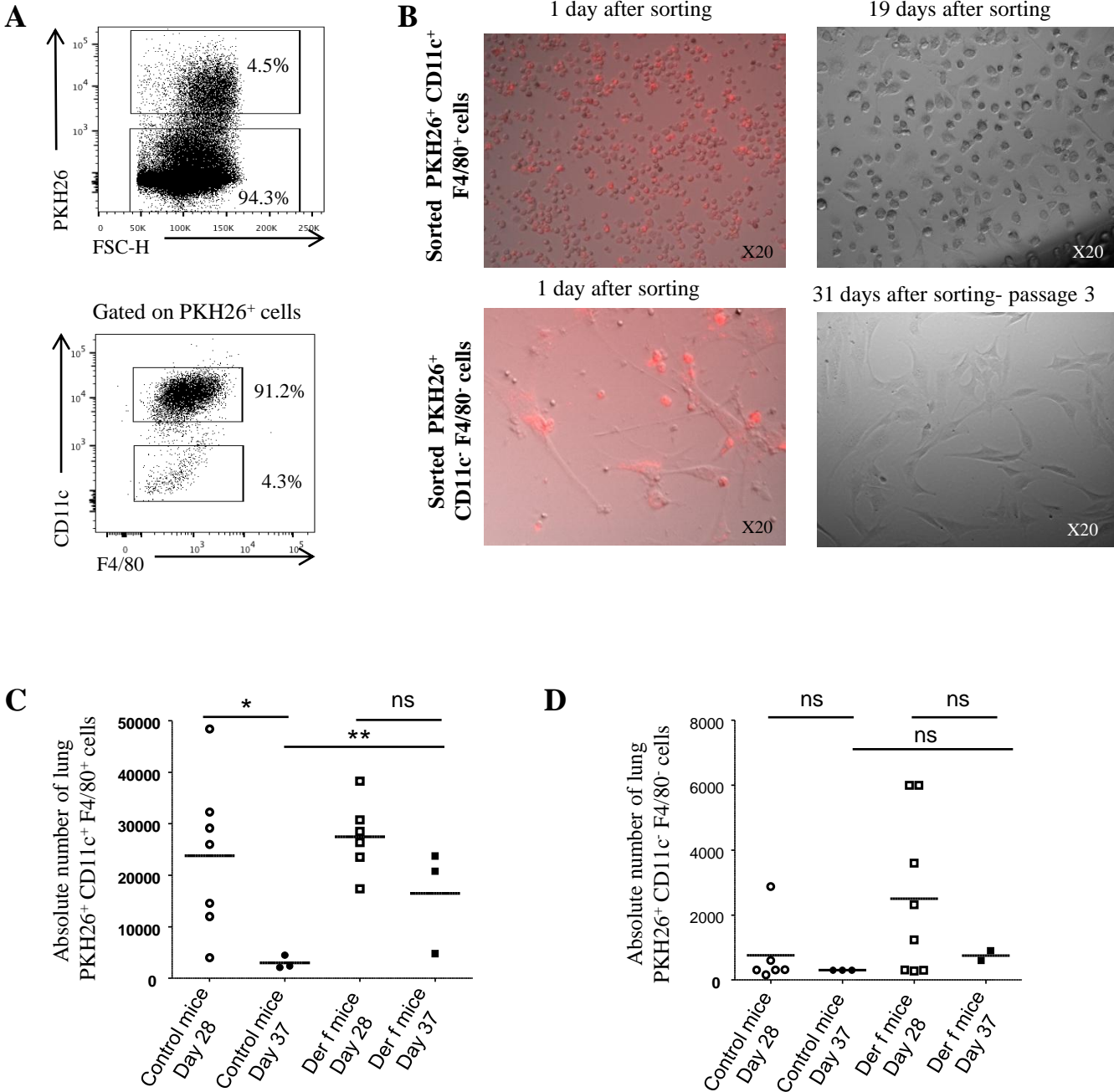
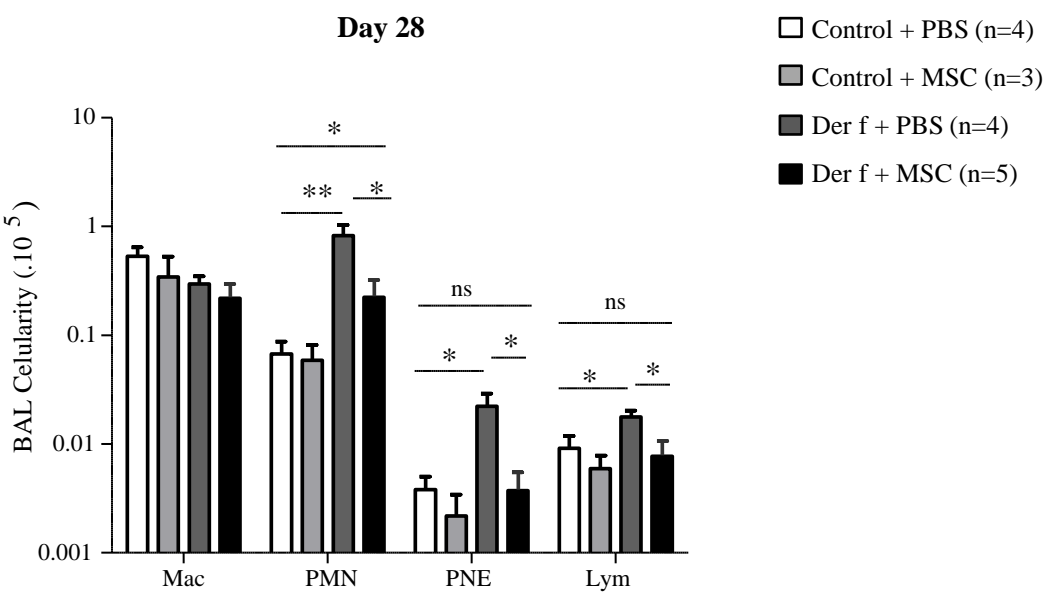


Figure 5

A



B

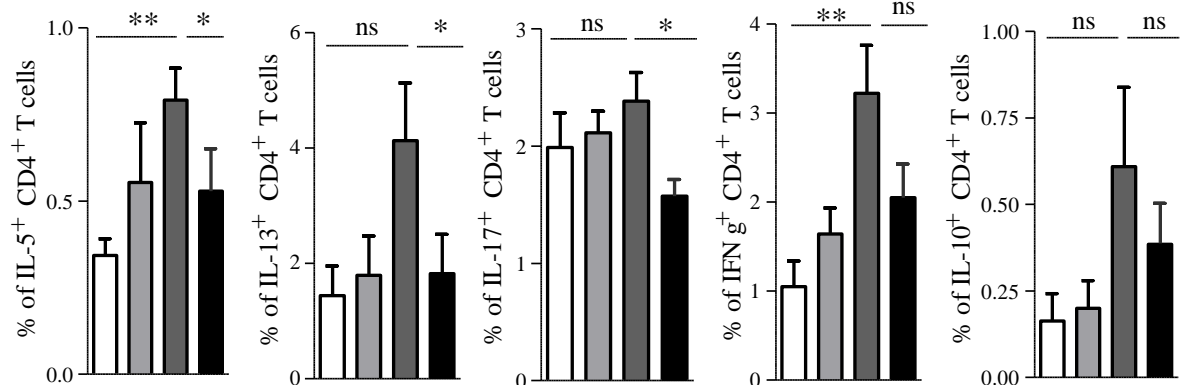
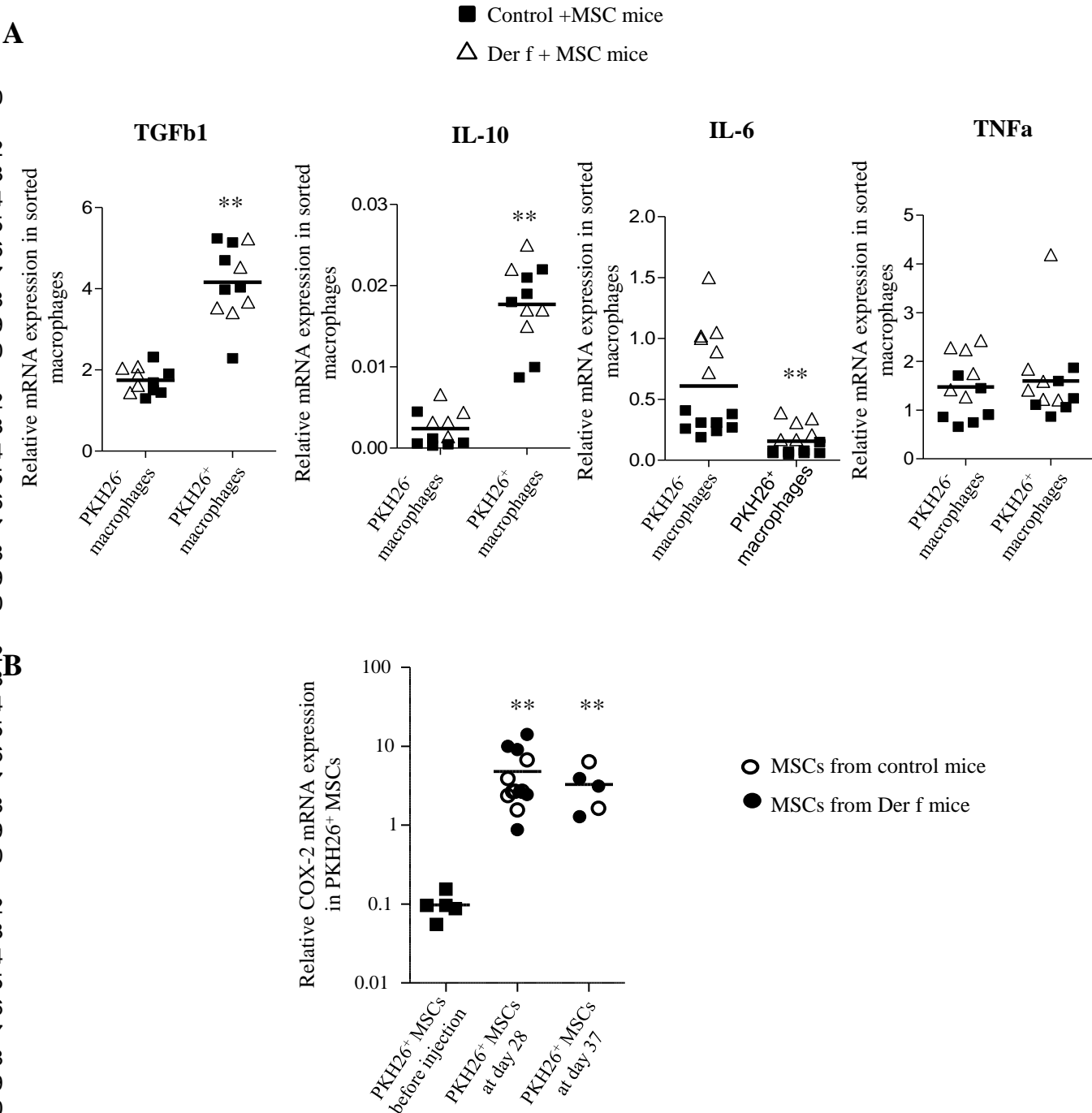
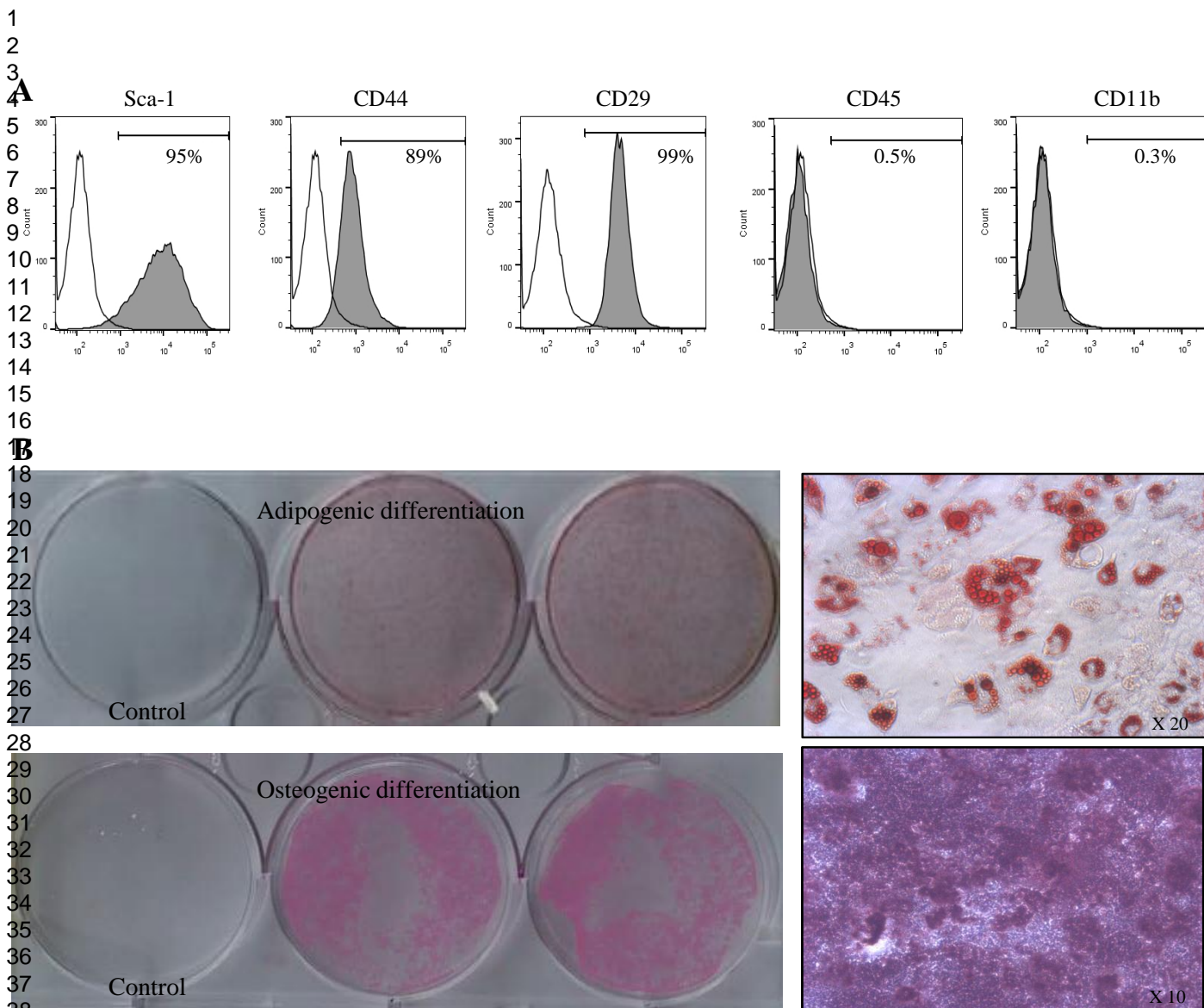


Figure 6

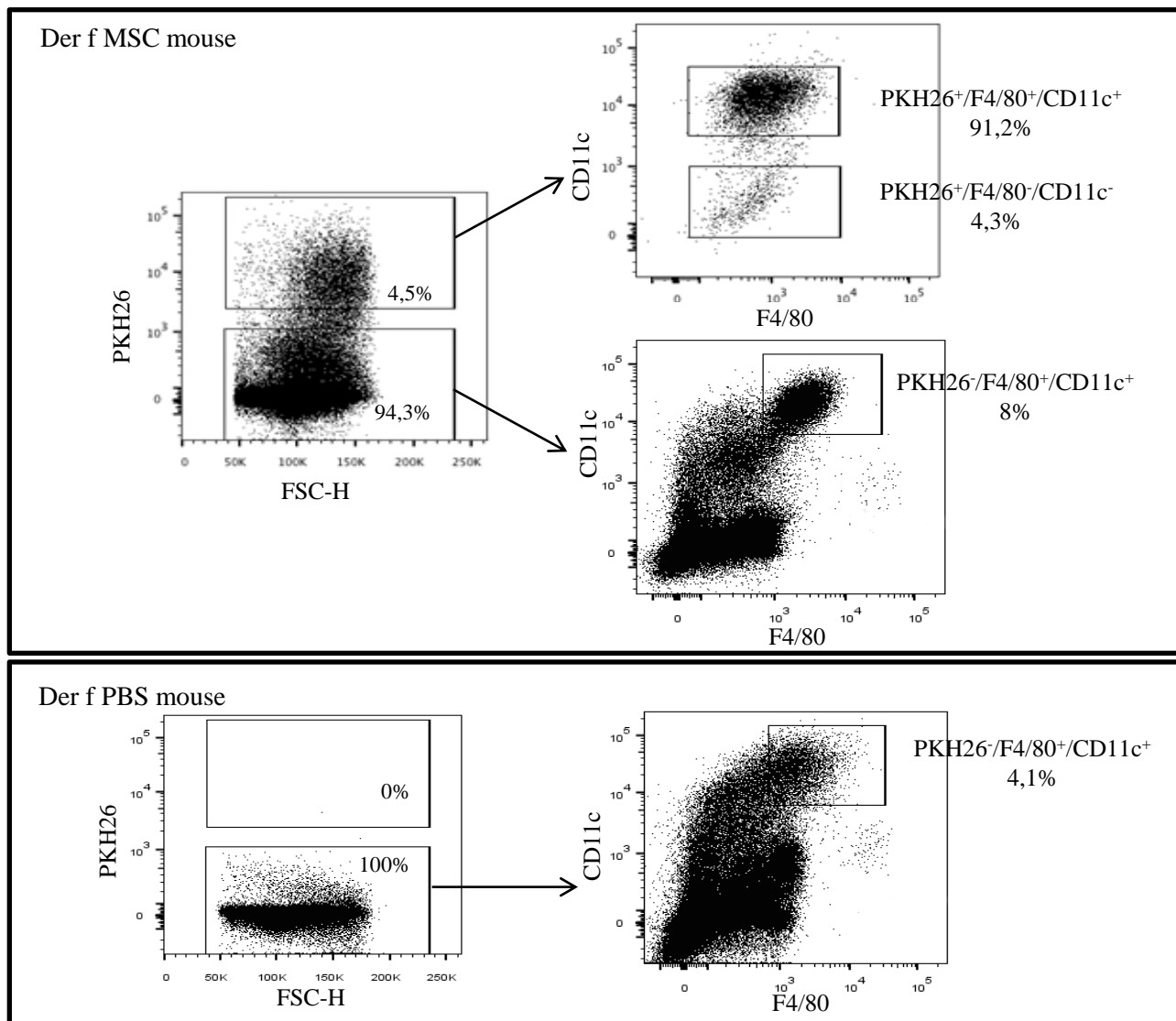
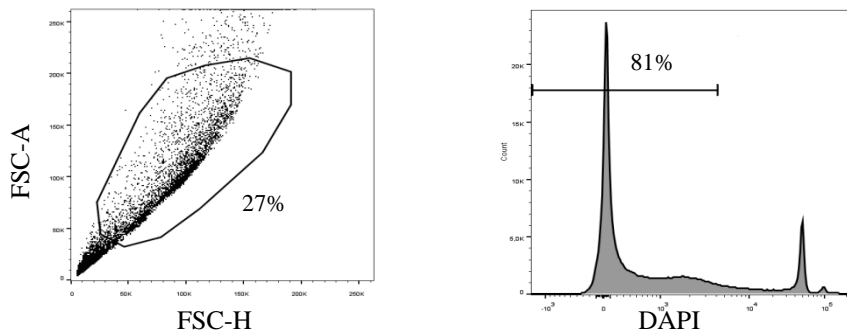


Supplementary Figure 1



Supplementary Figure 1 : Mesenchymal stem cell characterization.

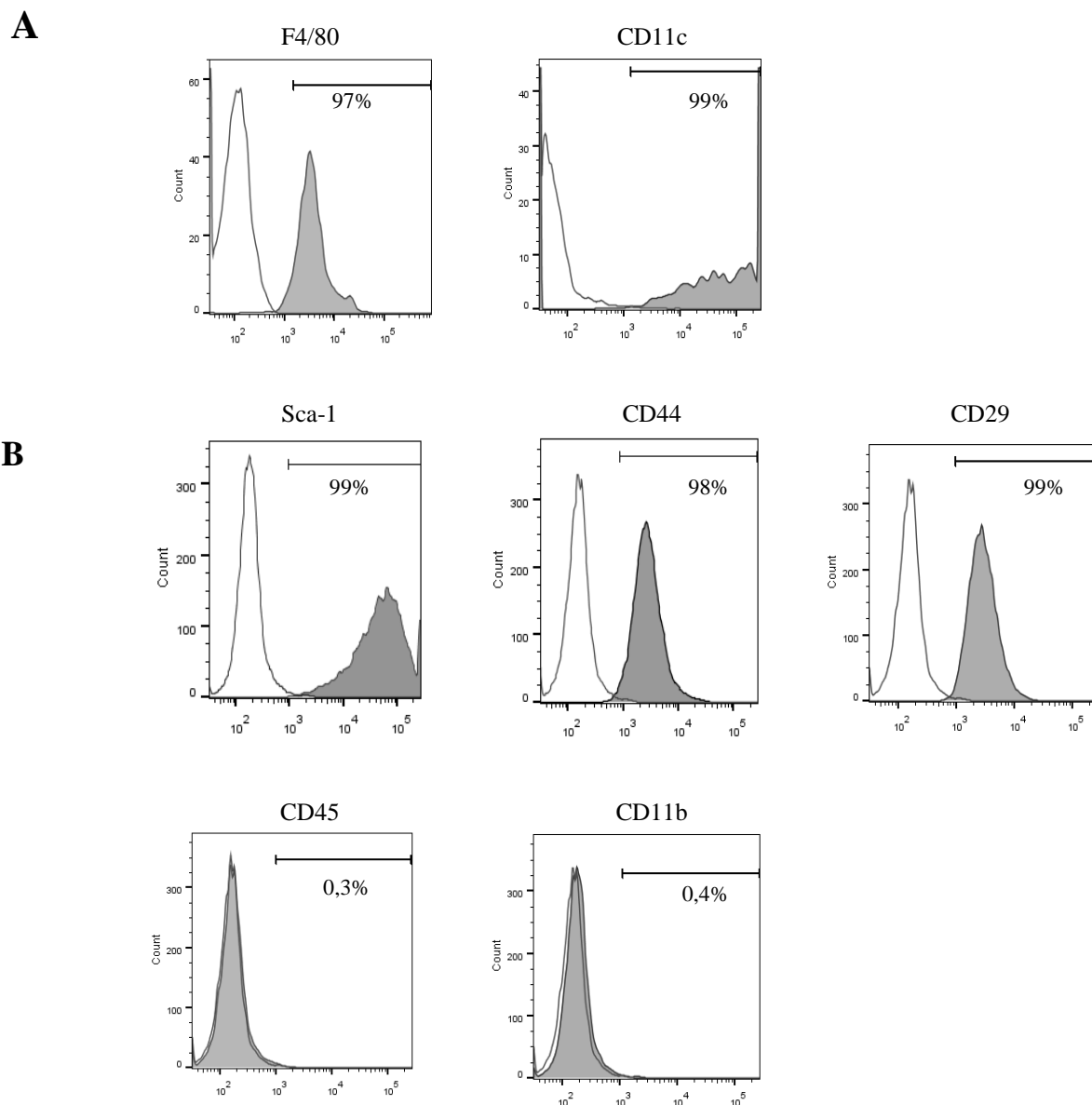
After passage 8, MSCs isolated from BALB/c bone marrow were characterized by flow cytometry, and by their ability to adipogenic and osteogenic differentiation. (A) Specific MSC phenotype was distinguished by Sca-1, CD29, CD44 positive expression, and CD45, CD11b negative expression. (B) MSC adipogenic differentiation was characterized by fat drops revealed by Oil Red O labeling. MSC osteogenic differentiation was characterized by calcium precipitate revealed by Alizarin Red S labelling. Controls are MSCs cultured in MSC medium.



Supplementary Figure 2 : Strategy of gating for cells sorting by flow cytometry.

Representative images for cell isolation by flow cytometry at day 28 in Der f + MSC and Der f + PBS mice. Cells were sorted after gating for morphology (FSC-H vs FSC-A) and viability (negative for DAPI). On these gates, cells PKH26⁺ and PKH26⁻ were identified and further selected for cells sorting by F4/80 and CD11c expression.

Supplementary Figure 3

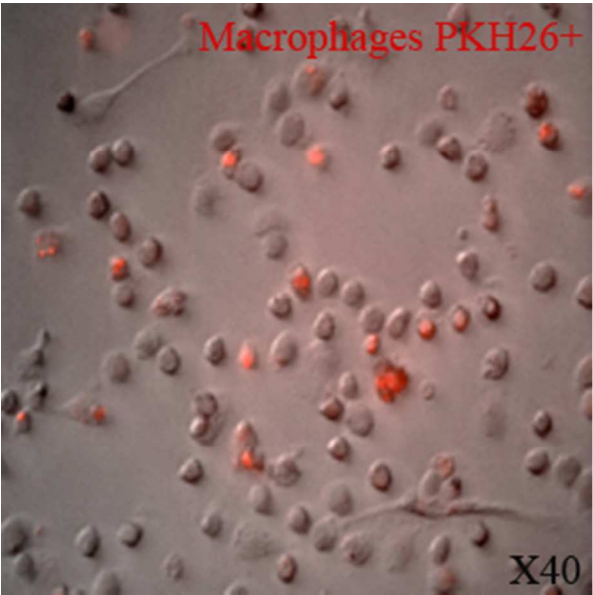


Supplementary Figure 3 : Sorted cell characterization after *in vitro* culture

(A) After 19 days of culture PKH26⁺CD11c⁺F4/80⁺ cells sorted by flow cytometry expressed macrophage specific markers (F4/80, CD11c) confirming that these cells were macrophages.

(B) After 31 days of culture and 3 passages, PKH26⁺ CD11c⁻ F4/80⁻ cells sorted by flow cytometry showed MSC specific phenotype (Sca-1⁺, CD44⁺, CD29⁺, CD45⁻, CD11b⁻) confirming that these cells were the injected PKH26⁺ MSCs.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



49x49mm (150 x 150 DPI)